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Mutations of the APC tumor suppressor gene are linked to a number of hyperplastic events in humans and in mice, including breast cancer. Recent evidence indicates that the APC gene product may play an active role in the **wnt-1** signaling pathway as a regulator of cytoplasmic B-catenin, a known mediator of transcription. Cytoplasmic B-catenin signaling may contribute to the transformation of cancerous cells, mediating the acquisition of a more aggressive, invasive phenotype. This project proposes to identify genes which may be regulated by B-catenin by using a novel method called gene-trapping, as well as through investigation of several candidate genes, including cadherin-11. Characterization of these genes will potentially elucidate another portion of the **wnt-1** signaling pathway, and give fresh insight into the exact nature of the cell cycle perturbations caused by B-catenin and other components of the pathway.

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Carol Feller 8-2
PI - Signature Date

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INTRODUCTION

Cell to cell adhesion is a phenomenon often affected in cancer. Important for everything from development to cellular communication, the mechanisms of adhesion may offer clues about the nature of metastasis, invasion, and cancer progression. One important class of cell adhesion molecules is that of the cadherins. These are a family of calcium-dependent transmembrane proteins that mediate cell-cell interactions through homotypic extracellular associations.¹ They are particularly important during cellular differentiation and morphogenesis. Anchoring cadherins to the actin cytoskeleton are a second class of proteins known as catenins, three of which have been identified: α -catenin, similar to the actin-binding protein vinculin; β -catenin, homologous to the *Drosophila* segment polarity gene Armadillo; and γ -catenin, or plakoglobin, found in adherens and desmosomal junctions.

β -catenin itself is a 92-kD protein that contains several conserved regions known as armadillo repeats.² Although originally identified as a link between E-cadherin and the actin-bound α -catenin, recent studies have established β -catenin's role as not only a cell adhesion molecule but also as a signaling molecule in the *wnt-1* pathway, with putative roles in both colon and breast cancer.^{3,4}

The *wnt-1* pathway, which is thought to be involved both in normal development and cancer, is still under investigation. It is believed that the *wnt-1* signal indirectly leads to the down-regulation of GSK3 β , a serine-threonine kinase. Normally, the absence of the *Wnt* signal allows GSK3 β to phosphorylate the APC gene-product, which in turn reduces cytoplasmic levels of β -catenin protein. It is thought that APC and GSK3 β function in concert to control cytoplasmic β -catenin levels by targeting the protein for degradation. In the presence of the *Wnt* signal, cytoplasmic β -catenin levels remain high. Many breast and colon cancer cell lines, due to both known and putative mutations in many of the molecules of this pathway, also exhibit high levels of cytoplasmic β -catenin. In addition, recent studies have found that β -catenin interacts with the TCF/LEF family

of known transcription factors.⁵ This, in addition to evidence that β -catenin accumulates in the nucleus when cytoplasmic levels are increased, has lead to speculation that β -catenin serves to regulate the expression of other gene products which may be important factors in the etiology of cancer at the cellular level.⁶

The goal of this study is to identify putative downstream targets of β -catenin, and to study how these gene products predispose cells to cancerous phenotypes. In order to do this, we have used a two-pronged approach: first, the application of the gene-trap technique; and second, the identification and investigation of several candidate targets, based upon previous studies. One of these, Cadherin-11, is very promising, and is being investigated vigorously.

Cadherin-11 is a member of the family of Type II cadherins, all homotypic cell adhesion molecules related to one another structurally. Originally identified as an adhesion molecule predominantly expressed in tissues of mesodermal origin,⁷ cadherin-11 has been subsequently identified as potentially relevant in the aggressive phenotypes of breast, colon, and renal cell carcinoma.^{8,9,10} The following results provide preliminary data to demonstrate that β -catenin signaling may play a role in regulating the expression of cadherin-11.

MATERIALS AND METHODS

Cell Culture. All cell lines used in these studies (MCF7, MDA-MB 231, MDA-MB 435, HS578T, SKBR3) were obtained from the ATCC (American Tissue Culture Core; Rockville, MD) and were maintained at 37°C, 5% CO₂ in DMEM containing 5% fetal bovine serum.

Transient Transfection. Cells were transfected with a full length β -catenin expression vector or a CAT control vector using Lipofectamine Plus (Life-Technologies).

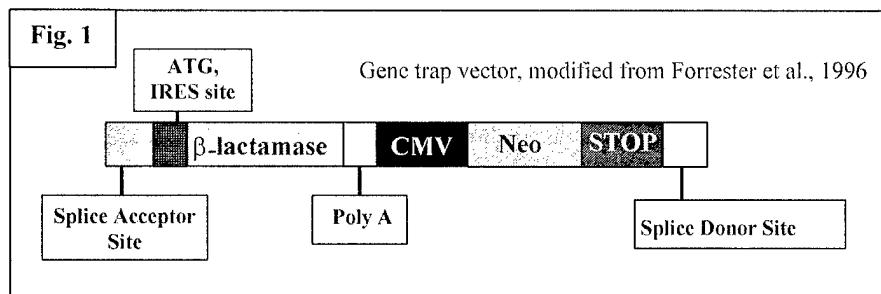
RT-PCR. cDNA was synthesized using MMLV-RT and reverse primers. Subsequent PCR was performed in a PE thermocycler.

Western Analysis. Cells were lysed using an NP-40 solution, and soluble fractions were separated in tris-glycine gels using electrophoresis. Proteins were subsequently transferred to nitrocellulose and probed with appropriate antibodies.

Antibodies. Antibodies to the intracellular domain of cadherin-11 were raised in collaboration with Zymed laboratories. After characterization of three monoclonal antibodies, 5B2H5 was used for subsequent experiments. β -catenin detection was performed using the Transduction Laboratory monoclonal antibody.

RESULTS

Gene Trap. As explained in the previous update, the original gene trap vectors provided

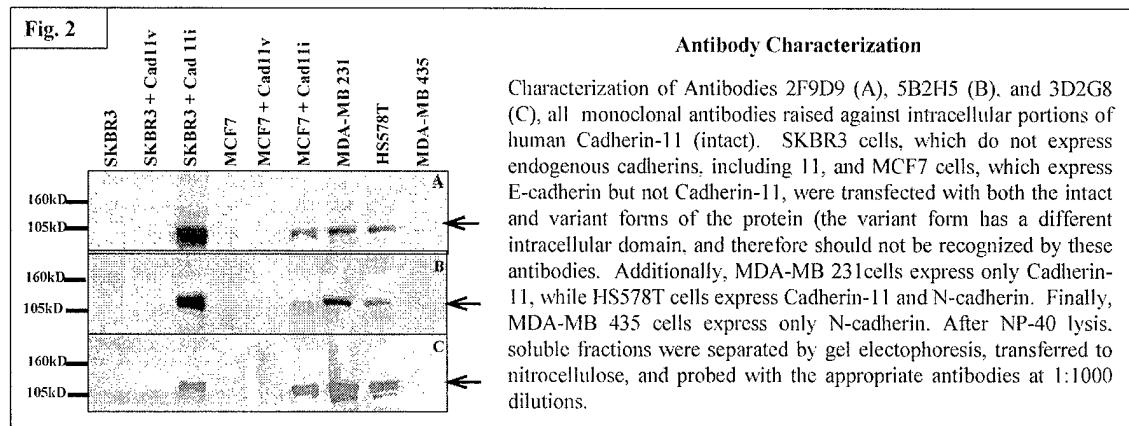


to us by LM
Forrester¹¹
contained the
lacZ reporter
gene.

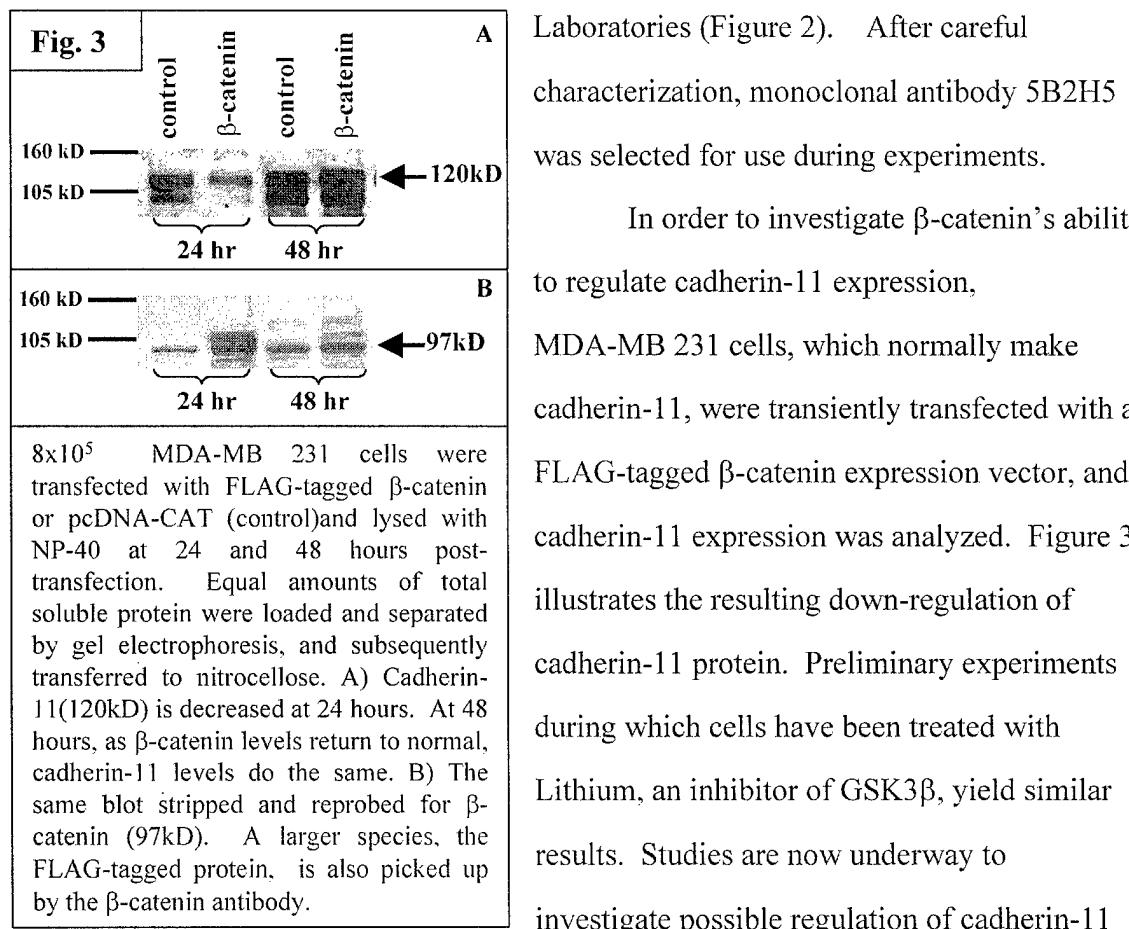
Reengineering of this vector (Figure 1) was performed in collaboration with Dr. Robert Lechleider[†] with the following results: insertion of an IRES site to minimize difficulties pertaining to potential internal ATG's in the trapped gene, use of a splice donor site to facilitate vector insertion, as well as a terminal stop codon. Most importantly, the *lacZ* reporter cassette was replaced with β -lactamase, which is a much more sensitive detection agent than GFP.¹² Cells are in the process of being stably transfected with this construct and are scheduled to be screened this fall. Analysis of trapped genes will follow. Technical difficulties concerning construction of this vector have caused this project to take more time than anticipated, resulting in the additional pursuit of the cadherin-11 studies as follows.

[†] Department of Pharmacology, Uniformed Services University of the Health Sciences; Bethesda, MD

Cadherin-11 As noted in the introduction, it has been previously published that



Cadherin-11 is of significant interest in breast cancer. In addition, previous research has suggested that β -catenin may contribute to the regulation of its expression.¹³ In order to facilitate detailed analysis of β -catenin's possible role, antibodies to the intracellular portion of cadherin-11 were designed and produced in collaboration with Zymed



by β -catenin at the RNA level. Further insight into this regulatory phenomenon, as well as mechanistic studies investigating cadherin-11's role in oncogenesis, will hopefully expand current knowledge of β -catenin's role as an oncogenic signal transducer.

CONCLUSIONS

These preliminary studies have resulted in evidence that β -catenin may indeed regulate the expression of cadherin-11 in breast cancer cells. Further experiments planned include examination of this regulation at the RNA level, isolation and characterization of the cadherin-11 promoter, as well as functional studies pertaining to cadherin-11's role *in vivo*. Additional effort in this area as well as the gene-trap project constitute the work being undertaken as allowed by this grant.

¹ Pierceall, WE, AS Woodard, JS Morrow, D Rimm, and ER Rearon. 1995. Frequent alterations in E-cadherin and α - and β -catenin expression in human breast cancer cell lines. *Oncogene*. 1:1319-1326.

² Funayama, N, F Fagotto, P McCrea, and BM Gumbiner. 1995. Embryonic axis induction by the armadillo repeat domain of β -catenin: evidence for intracellular signalling. *J. Cell. Bio.* 128:959-968

³ Rubinfeld, B, I Albert, E Porfiri, C Fiol, S Munemitsu, and P Polakis. 1996. Binding of GSK3 β to the APC-- β -catenin complex and regulation of complex assembly. *Science*. 272:1023-1026.

⁴ Gumbiner, BM. 1995. Signal transduction by β -catenin. *Curr. Opin. Cell Bio.* 7:634-640.

⁵ van de Wetering, M, R Cavallo, D dooijes, M wan Beest, J va Es, J Loureiro, A Ypma, D Hursh, T Jones, A Bejsovec M Peifer, M Martín and H Clevers. 1997. Armadillo coactivates transcription driven by the product of the drosophila segment polarity gene *dTCF*. *Cell*. 88:789-799.

⁶ I Simcha, M Shtutman, D Saloman, J Zhurinsky, E Sadot, B Geiger, and A Ben-Ze'ev. 1998. Differential nuclear translocation and transactivation potential of β -catenin and plakoglobin. *J. Cell Bio.* 141:1433-1448.

⁷ Hoffmann, I, and R Balling. 1995. Cloning and expression analysis of a novel mesodermally expressed cadherin. *Dev. Bio.* 169:337-346.

⁸ Pishvaian, MJ, CM Feltes, P Thompson, MJ Bussemakers, JA Schalken, and SW Byers. 1999. Cadherin-11 is expressed in invasive breast cancer cell lines. *Canc. Res.* 59:947-952.

⁹ Munro, SB, IM Turner, R Farookhi, OW Blaschuk, and S Jothy. 1995. E-cadherin and OB-cadherin mRNA levels in normal human colon and colon cancer. *Exp. Mol. Path.* 62: 118-122.

¹⁰ Shimazui, T, LA Giroldi, PP Bringuier, E Oosterwijk, and JA Schalken. 1996. Complex cadherin expression in renal cell carcinoma. *Canc. Res.* 56:3234-3237.

¹¹ Forrester, LM, A Nagy, M Sam, A Watt, L Stevenson, A Bernstein, AL Joyner, and W Wurst. 1996. An induction gene trap screen in embryonic stem cells. *Proc. Natl. Acad. Sci.* 93:1677-1682.

¹² Whitney, M, E Rockenstein, G Cantin, T Knapp, G Zlokarnik, P Sanders, K Durick, FF Craig, PA Negulescu. 1998. A genome-wide functional assay of signal transduction in living mammalian cells. *Nat. Biotech.* 16:1329-1333.

¹³ Hadeball, B, A Borchers, D Wedlich. 1998. Xenopus cadherin-11 (Xcadherin-11) expression requires the Wg/Wnt signal. *Mech. Dev.* 72:101-113

Key Research Accomplishments:

- Construction of new gene-trap vector
- Preparation of cell lines (i.e. stable transfection) with gene-trap vector
- Design and production of specific monoclonal antibodies against cadherin-11
- Analysis of cadherin-11 profile in invasive breast cancer cells
- Preliminary data supporting β -catenin's role in regulation of cadherin-11 expression

Reportable Outcomes

- Development of specific monoclonal antibodies against cadherin-11 (in collaboration with Zymed Laboratories). These antibodies are being made commercially available.
- Second author manuscript:
 - Pishvaiaiin, MJ, CM Feltes, P Thompson, MJ Bussemakers, JA Schalken, and SW Byers. 1998. Cadherin-11 is expressed in invasive breast cancer cell lines. *Canc. Res.* 59:947-952.

Cadherin-11 Is Expressed in Invasive Breast Cancer Cell Lines¹

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ABSTRACT

In several cancers, including breast cancer, loss of E-cadherin expression is correlated with a loss of the epithelial phenotype and with a gain of invasiveness. Cells that have lost E-cadherin expression are either poorly invasive with a rounded phenotype, or highly invasive, with a mesenchymal phenotype. Most cells lacking E-cadherin still retain weak calcium-dependent adhesion, indicating the presence of another cadherin family member. We have now examined the expression of the mesenchymal cadherin, cadherin-11, in breast cancer cell lines. Cadherin-11 mRNA and protein, as well as a variant form, are expressed in the most invasive cell lines but not in any of the noninvasive cell lines. Cadherin-11 is localized to a detergent-soluble pool and is associated with both α - and β -catenin. Immunocytochemistry shows that cadherin-11 is localized to the cell membrane at sites of cell-cell contact as well as at lamellipodia-like projections, which do not interact with other cells. These results suggest that cadherin-11 expression may be well correlated with the invasive phenotype in cancer cells and may serve as a molecular marker for the more aggressive, invasive subset of tumors. Cadherin-11 may mediate the interaction between malignant tumor cells and other cell types that normally express cadherin-11, such as stromal cells or osteoblasts or perhaps even with the surrounding extracellular matrix, thus facilitating tumor cell invasion and metastasis.

INTRODUCTION

Cadherins are transmembrane adhesion molecules that mediate calcium dependent cell-cell adhesion. The cadherins are members of a superfamily of related proteins, the members of which include the "classical" cadherins, desmosomal cadherins, protocadherins, and products of tumor suppresser genes like *c-ret* and *Fat* (1). Members of the classical cadherin subgroup have been shown to be essential for strong cell-cell adhesion and maintenance of tissue integrity and cell polarity (2). They also facilitate, via homophilic adhesion, the differential sorting of cells during morphogenesis (3). Cadherin function is mediated by its connections with the cytoplasmic catenins, α , β , and γ (4, 5). The catenins link cadherins to the actin cytoskeleton and have other signaling functions as well (6).

The original cadherins (E-, P-, and N-cadherin) were defined based upon their expression patterns in the adult (7). More recently, many other cadherins have been identified, and the classical cadherin subgroup has been subdivided into two groups (8–10): the type I cadherins, which include E-, P-, and N-cadherin; and the type II cadherins, which include cadherins-5, -6, -7, -8, -9, -10, -11, and -12. Type I cadherins share common structural features but low amino acid homology with the type II cadherins (9, 10). The function and expression patterns of the type I cadherins have been studied extensively, but little is known to date about the type II cadherins (3).

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¹ This work was funded by grants from the United States Department of Defense and Adherex Technologies (to S. W. B.). M. J. P. and C. M. F. are recipients of graduate fellowships from the Department of Defense.

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Previously, it was shown in a panel of breast cancer cell lines that E-cadherin expression is lost as cells become more invasive and less differentiated (11, 12). Loss of E-cadherin is also associated with the less differentiated, more invasive subset of breast tumors (1). However, exogenous expression of E-cadherin in invasive breast cancer cells did not inhibit their invasive phenotype (11). In addition, blockade of E-cadherin in noninvasive cells prevented cell-cell adhesion but did not result in an invasive phenotype (12). Instead, E-cadherin expression and the state of differentiation of these cells is representative of their place along a putative epithelial-mesenchymal transition (11, 12). As the cells become more fibroblastoid, they acquire the molecular characteristics of fibroblasts, including an increase in vimentin expression, and loss of known junctional proteins including E-cadherin, desmoplakin, and the tight junctional protein, ZO-1 (Table 1). Nevertheless, even the most invasive cell lines exhibited calcium-dependent cell-cell adhesion, suggesting the presence of another functional cadherin (12). Recent work has demonstrated the presence of other cadherins in mesenchymal and fibroblast cells (13, 14). One such mesenchymal cadherin is cadherin-11 (15). We now show that cadherin-11 mRNA and protein and a cadherin-11 variant mRNA are expressed in invasive and poorly differentiated breast cancer cell lines. Cadherin-11 is localized to the cell membrane in a detergent-soluble complex, where it associates with α -, and β -catenin, and may facilitate tumor cell invasion and metastasis.

MATERIALS AND METHODS

Cell Lines. All cell lines were obtained from American Type Culture Collection and grown in DMEM (Life Technologies, Inc.) plus 5% fetal bovine serum as described previously (11). A summary of the characteristics of the breast cancer cell lines has been published (11). An expanded table is included below (see Table 1). Please note in particular the level of invasiveness of each cell line, as well as the expression of the various adherens junction molecules. MCF-7_{ADR} cells are a variant of MCF-7 cells that are resistant to Adriamycin.

RT-PCR.³ RT-PCR was performed using 0.2 μ g (β -actin) or 1.0 μ g (cadherin-11) of total RNA, isolated using the guanidinium isothiocyanate method (16). The following primers were used: β -actin upstream, 5'-TGACGGGTACCCACACTGTGCCCATCTA-3'; β -actin downstream, 5'-CTAGAACGATTGCGGTGGACATGGAGGG-3'; cadherin-11 wild-type upstream, 5'-ACCAGATGCTGTGTCAGA-3'; cadherin-11 wild-type downstream, 5'-GTCATCCTTGTCTCATCTGCA-3'; cadherin-11 variant upstream, 5'-CGCCGCGGATCCTTAATGGAACCCCCCTCTC-3'; and cadherin-11 variant downstream, 5'-CCGCCGGAATCTCCGTAAGTGTGGTTGGACTCTC-3'. First-strand synthesis with the downstream primer and MMLV-RT (Gibco/BRL) was followed by PCR using Taq polymerase (Life Technologies, Inc.) after adding the upstream primer. The following cycling parameters were used: cadherin-11 wild type, 94°C for 30 s, 55°C for 30 s, 72°C for 2 min, 35 cycles; cadherin-11 variant, 94°C for 1.5 min, 55°C for 2 min, 72°C for 3 min, 35 cycles. Both parameters could be used for β -actin. The PCR product was run on a 1% agarose gel. The following fragments were amplified: β -actin, a 661-bp fragment that spans an intron to rule out genomic contamination; cadherin-11 wild type, a 742-bp fragment from a region encoding part of the extracellular domain; cadherin-11 variant, a 194-bp fragment that encodes most of the COOH-terminal 75 amino acids present only in the variant (17).

³ The abbreviations used are: RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 1 Molecular characteristics of several breast cancer cell lines

This is based on a table published previously by Sommers *et al.* (12). Data on N-cadherin protein is from Hazan *et al.* (19). Unless specified otherwise, the data represent the presence or absence of mRNA and protein, using the following scale: 0, negative; 1, +/−; 2, +; 3, ++; 4, +++; ND, not determined.

| Cell line | Matrikel | Invasion | ZO-1 | DP-I/II | E-cadherin | α-Catenin | β-Catenin | Plak ^a | NcadProt | NcadRNA | Cad11 WT | Cad11 var | Vimentin |
|----------------------|-----------|----------|------|---------|------------|-----------|-----------|-------------------|----------|---------|----------|-----------|----------|
| BT483 | Fused | 2 | 3 | 3 | 2 | 2 | 2 | ND | ND | ND | ND | ND | 0 |
| MDA-MB-175-7 | Fused | 2 | 3 | 2 | 2 | 2 | 2 | ND | 1 | 0 | 0 | ND | 0 |
| MCF-7 | Fused | 2 | 3 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | ND | 0 |
| ZR-75-B | Fused | 2 | 3 | 2 | 2 | 2 | 2 | ND | 1 | 0 | 0 | 0 | 0 |
| T47D | Fused | 2 | 2 | 2 | 2 | 2 | 2 | ND | 1 | 0 | 0 | 0 | 0 |
| BT474 | Fused | 2 | 0 | 2 | 2 | 2 | 2 | 0 | 2 | 0 | 0 | 0 | 0 |
| MDA-MB-361 | Fused | 2 | 0 | 2 | 2 | 2 | 2 | 0 | ND | ND | ND | ND | 0 |
| MDA-MB-468 | Fused | 2 | 0 | 2 | 1 | 0 | 1 | 1 | 0 | 2 | 0 | 0 | 0 |
| SK-BR-3 | Spherical | 2 | 2 | 1 | 0 | 2 | 1 | 1 | 0 | ND | 0 | 0 | 0 |
| CAMA-1 | Spherical | 2 | 2 | 2 | 0 | 1 | 1 | 1 | ND | 1 | 0 | 0 | 0 |
| MDA-MB-453 | Spherical | 2 | 0 | 1 | 0 | 2 | 1 | 1 | 0 | 3 | 0 | 0 | 0 |
| MDA-MB-134 | Spherical | 2 | 0 | 1 | 0 | 1 | 1 | 1 | ND | 3 | 0 | 0 | 0 |
| MDA-MB-436 | Stellate | 3 | 2 | 0 | 0 | 2 | 2 | 1 | 2 | 3 | 0 | 0 | 2 |
| MDA-MB-435 | Stellate | 3 | 0 | 0 | 0 | 2 | 4 | ND | 2 | 3 | 0 | 0 | 2 |
| MCF-7 _{ADR} | Stellate | 3 | 0 | 0 | 0 | 2 | 2 | 1 | ND | 3 | 2 | 2 | 2 |
| MDA-MB-157 | Stellate | 3 | ND | ND | 0 | 0 | 3 | ND | ND | ND | 2 | 2 | ND |
| MDA-MB-231 | Stellate | 4 | 0 | 0 | 0 | 2 | 2 | 1 | 0 | ND | 3 | 2 | 2 |
| BT549 | Stellate | 4 | 0 | 0 | 0 | 2 | 2 | 1 | 2 | ND | 3 | 2 | 2 |
| HS578T | Stellate | 4 | 0 | 0 | 0 | 2 | 2 | 0 | 2 | 3 | 3 | 2 | 2 |

^a Plak, Plakoglobin; NcadProt, N-cadherin protein; NcadRNA, N-cadherin RNA; Cad11 WT, cadherin-11 wild-type; Cad11 var, cadherin-11 variant.

Northern Blot. Twenty μg of total RNA were separated on a 1% agarose gel and transferred to a nylon membrane (Boehringer Manheim; Ref. 16). A 1.6-kb fragment of the cadherin-11 cDNA was labeled using ³²P-labeled dCTP and used to probe the blot (DNA fragment donated by Colin MacCalman, University of British Columbia, Vancouver, British Columbia, Canada). The blot was hybridized at 50°C overnight, then washed three times in 2% SSC at 55°C and 65°C (last wash). The labeled bands were visualized using a phosphorimager. The nylon was then reprobed for GAPDH as a control.

Western Blot. Cells from confluent 10-cm dishes were isolated and sonicated homogenized in a hypotonic solution (10 mM Tris, 0.2 mM MgCl₂, pH 7.5). The homogenate was centrifuged first for 10 min at 3000 × g to remove nuclei. The supernatant was then centrifuged at 150,000 × g for 1 h. The supernatant of this centrifugation, defined as the cytoplasmic fraction, was added to four volumes of ethanol, and the proteins precipitated overnight. The proteins were then collected by ultracentrifugation and solubilized in sample buffer [2% SDS, 60 mM Tris (pH 6.8), and 10% glycerol]. The pellet from the first centrifugation was solubilized in a 1% NP40 buffer (1% NP40, 150 mM NaCl, and 50 mM Tris, pH 8.0) for 30 min and clarified in a microcentrifuge for 15 min. The resulting supernatant is the NP40 soluble membrane fraction. The pellet is the NP40 insoluble fraction, representing cytoskeletally associated proteins. Both were solubilized in sample buffer. Protein content in the samples was measured (Bio-Rad).

Proteins were separated on an 8% reducing polyacrylamide minigel (Novex), transferred to nitrocellulose (Protran), and blocked overnight in 5% milk. The blot was then probed with a monoclonal antibody to cadherin-11 (cad113H; ICOS Corp.). This antibody recognizes the extracellular domain of cadherin-11. A secondary peroxidase labeled antibody (Kirkegaard and Perry) was added, and the bands were visualized by ECL (Amersham). The blots were then stripped at 50°C for 30 min [stripping solution: 62.5 mM Tris (pH 7.5), 2% SDS, and 1.7% (v/v) β-mercaptoethanol] and sequentially reprobed, first with a pan-cadherin polyclonal antibody (Sigma), next with a monoclonal antibody to E-cadherin (Transduction Labs), and finally with antibodies against α-catenin (polyclonal from David Rimm, Yale University, New Haven, CT) or β-catenin (monoclonal antibody; Transduction Labs).

Immunoprecipitation and Immunocytochemistry. For immunoprecipitation, cells from a confluent 10-cm dish were lysed in a 1% NP40 buffer (see above). The lysate was clarified in a microcentrifuge, and the supernatant was precleared with 100 μg of normal rabbit serum. The lysate was immunoprecipitated at 4°C overnight using a polyclonal antibody against either α- or β-catenin (both donated by David Rimm, Yale University). The bound proteins were isolated with protein A-Sepharose beads (Boehringer Manheim), washed six times, and boiled in Laemmli buffer with β-mercaptoethanol, and the Western blot was performed as described above (18).

For immunocytochemistry, cells were grown on 18-mm coverslips, then fixed in 2% paraformaldehyde for 15 min, and blocked in 3% ovalbumin for 1 h at room temperature. Coverslips were incubated in primary antibody

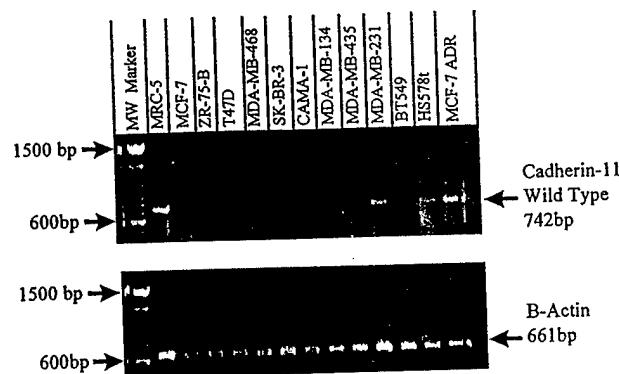
(cadherin-11 monoclonal antibody or a β-catenin polyclonal antibody) overnight at 4°C and then washed and incubated with Texas Red-conjugated secondary antibody for 1 h at room temperature. Coverslips were washed, mounted using Vectashield fluorescence mounting medium (Vector), and visualized on a Zeiss microscope.

RESULTS

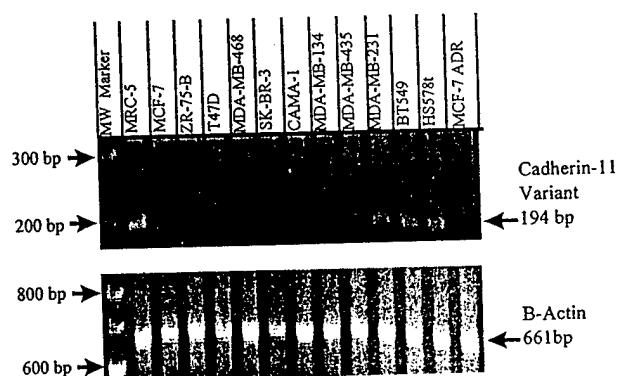
Cadherin-11 Wild-Type and Variant mRNA Expression in Human Breast Cancer Cell Lines. We first examined the expression of cadherin-11 in breast cancer cell lines by RT-PCR. Two sets of primers were used. The first amplifies a region that encodes the extracellular domain of cadherin-11 and thus would recognize both the wild-type and variant cadherin-11; the second set amplifies only a portion of the variant cadherin-11 (17). β-actin primers were used as a control. MRC-5, a human embryonic lung fibroblast cell line known to express cadherin-11, was used as a positive control (19). Cadherin-11 wild-type (Fig. 1a) and variant (Fig. 1b) mRNA were expressed in five invasive cell lines, MDA-MB-157 (RT-PCR and Northern not shown), MDA-MB-231, BT549, HS578T, and MCF-7_{ADR}. In MCF-7_{ADR} cells, which are considerably less invasive than the others, the variant is expressed at very low levels. Two invasive cell lines, MDA-MB-436 and MDA-MB-435, did not express cadherin-11 nor did any of the noninvasive cell lines. Although a variant cadherin-11 band was amplified from SK-BR-3 cell mRNA, we do not believe that this result represents true expression of cadherin-11, considering that all other results were negative (wild-type RT-PCR, Northern and Western blots). The RT-PCR results were confirmed by Northern blot using a 1.6-kb fragment of the cadherin-11 cDNA as a probe (Fig. 1c). The probe identified a 4.4-kb band from the same five cell lines. Several larger bands can also be seen, but these have yet to be identified. The variant mRNA contains an insertion of 179 bp, but the Northern blot did not allow us to differentiate between wild-type and variant transcripts. MDA-MB-157 (not shown) and MCF-7_{ADR} cells express much lower levels of mRNA than MDA-MB-231, BT549, or HS578T cells, despite the presence of similar levels of total RNA and GAPDH expression.

Cadherin-11 Protein Expression. We next wanted to examine cadherin-11 protein expression. Ten μg of protein from a detergent lysate were run on a polyacrylamide gel, and the subsequent blot was probed with an anti-cadherin-11 monoclonal antibody (ICOS, Inc.; Fig. 2a). This monoclonal antibody recognizes the extracellular por-

A



B



C

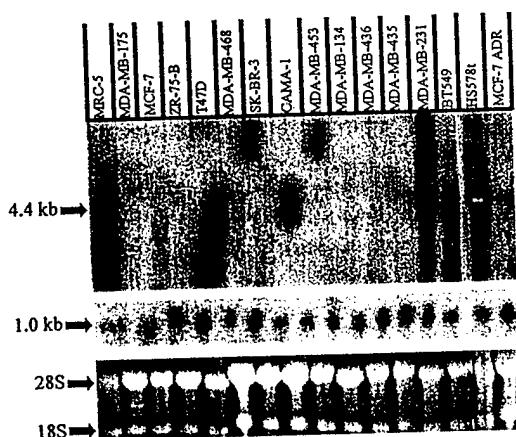


Fig. 1. Cadherin-11 mRNA expression RT-PCR and Northern blot analysis show that cadherin-11 mRNA is expressed in only the most invasive cell lines. In A and B, 1 μ g cadherin-11 mRNA (and variant) or 0.2 μ g (B-actin) of total RNA was used for the reaction. The primers amplified the following fragments: cadherin-11 wild-type, 742 bp (A, upper); cadherin-11 variant, 194 bp (B, upper); B-actin, 661 bp (A and B, lower). In C, 20 μ g of total RNA were run on an agarose gel and transferred to nylon. The membrane was probed sequentially with a cadherin-11 cDNA fragment (upper) or a GAPDH cDNA probe (middle). The ethidium bromide-stained agarose gel shows the 28S and 18S RNA bands clearly (lower).

tion of cadherin-11 and should recognize both wild-type and variant protein. In Western blots, cadherin-11 runs as a M_r ~120,000 band. The cell lines that contained cadherin-11 mRNA also expressed cadherin-11 protein. MDA-MB-157 and MCF-7_{ADR} cells express lower levels of cadherin-11 protein. Although the putative variant protein would be M_r ~7,000 smaller than the wild type, a band of this size could not be definitively identified on this Western blot (17). Several bands significantly smaller than the full-length cadherin-11 are present. One of these could represent the variant form if the protein migrated faster than expected. Alternatively, these bands could represent degradation products. The Western blot was reprobed

several times to examine the expression of other adherens junction proteins. Probing with a pancadherin antibody revealed that a number of cell lines express another member of the cadherin family (Fig. 2b). The pancadherin antibody is immunoreactive to several members of the cadherin family (the arrow indicates the location of the cadherin-11 band) but is only weakly reactive to E-cadherin. Based upon the strong immunoreactivity and molecular weight (M_r ~140,000), we believe the pancadherin antibody is identifying N-cadherin in most cell lines. This is consistent with results demonstrated by Hazan *et al.* (20). Interestingly, an anti-E-cadherin Western blot demonstrates that E-cadherin and cadherin-11 are never coexpressed (Fig. 2c). Finally, most cell lines express α -catenin (Fig. 2d) and β -catenin (Fig. 2e).

Cadherin-11 Protein Is Expressed Primarily in an NP40 Soluble Pool. We next wanted to determine the solubility of the cadherin-11 protein complex. Cultured cells were separated into three fractions (see "Materials and Methods"): cytoplasmic, NP40-soluble (membrane-bound, noncytoskeletally associated), and NP40-insoluble (cytoskeletally associated). Twenty-five μ g of protein from each fraction were run on a polyacrylamide gel, and the blotted proteins were probed with the cadherin-11 monoclonal antibody. In the cells expressing cadherin-11, cadherin-11 protein is found mostly in the

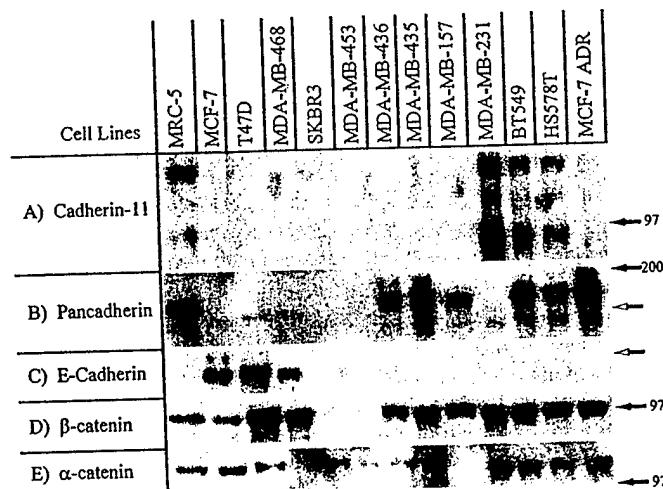


Fig. 2. Cadherin-11 Western blot. Ten μ g of protein from an NP40-soluble pool were run on PAGE. The subsequent nitrocellulose blot was probed with the following antibodies: A, cadherin-11 monoclonal antibody; B, pancadherin polyclonal antibody; C, E-cadherin monoclonal antibody; D, β -catenin monoclonal antibody; and E, α -catenin polyclonal antibody. The open arrow marks the location of the cadherin-11 band on the pancadherin and E-cadherin blots.

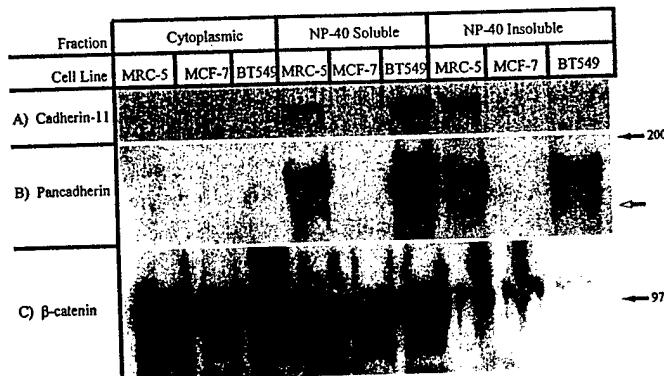


Fig. 3. Cadherin-11 Western blot-three pool. Twenty-five μ g of protein were run on SDS-PAGE. The subsequent nitrocellulose blot was probed with the following antibodies: A, cadherin-11 monoclonal antibody; B, pancadherin polyclonal antibody; and C, β -catenin monoclonal antibody. The open arrow marks the location of the cadherin-11 band on the pancadherin blot.

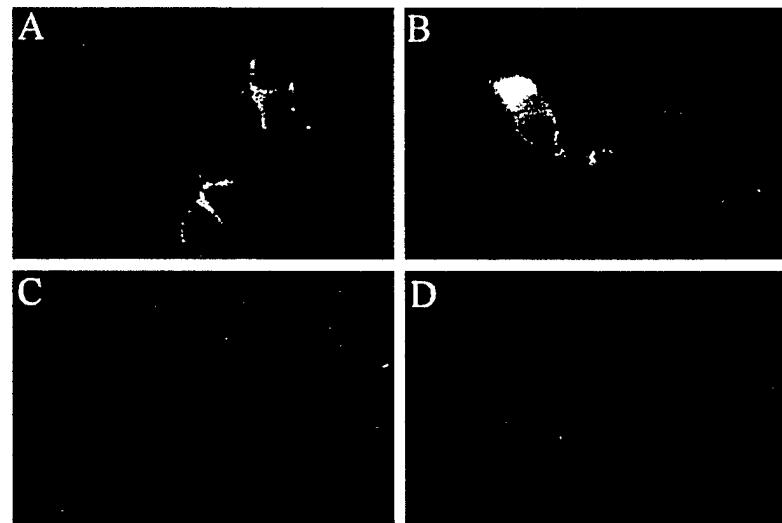


Fig. 4. Cadherin-11 immunocytochemistry. Cells were seeded on coverslips and fixed in paraformaldehyde. The fixed cells were stained with a cadherin-11 monoclonal antibody (A, B, and D) or with normal mouse IgG (C). Secondary staining with appropriate Texas Red-labeled goat antibodies revealed cadherin-11 expression in BT549 cells (A) and HS578T cells (B) but not in MCF-7 cells (D). Cadherin-11 is expressed at sites of cell-cell contact but also at lamellipodial extensions and possibly at regions of contact with the substrate. Staining with normal mouse IgG revealed no nonspecific staining (C).

NP40-soluble pool. Some cadherin-11 could also be found in the NP40-insoluble pool (Fig. 3a). As expected, no cadherin-11 was found in the cytoplasmic pool. By contrast, significant amounts of another cadherin (probably N-cadherin) were found in both the soluble and insoluble pools (Fig. 3b). Also, β -catenin could be found in all three pools, although less was found in the insoluble pool (Fig. 3c).

Cadherin-11 Is Localized to the Cell Membrane. In epithelial cells, cadherins typically function as cell-cell adhesion proteins as part of an adherens junction (21). However, cadherin-11 mRNA has been found only in mesenchymal cells, which do not usually form adherens junctions (13, 15). Immunocytochemistry was performed on paraformaldehyde-fixed cells (Fig. 4). Cadherin-11 is expressed at the cell membrane in BT549 and HS578T cells (Fig. 4, a and b) but not in MCF-7 cells (Fig. 4d). Staining of BT549 with normal mouse IgG reveals no nonspecific staining (Fig. 4c). In BT549 and HS578T cells, cadherin-11 is localized to sites of cell-cell contact, as is typical of cadherin family members. Surprisingly, cadherin-11 is also found in lamellipodia-like extensions and possibly at regions of contact with the substrate.

α -Catenin and β -Catenin Immunoprecipitations. Previously, Shibata *et al.* (19) were able to isolate cadherin-11 by probing a cDNA library with radiolabeled β -catenin protein, thus establishing their interaction *in vitro*. We wanted to determine whether cadherin-11 interacts with α -catenin and β -catenin *in vivo* (Fig. 5). Confluent cells were lysed in an NP40 buffer, and the precleared lysate was immunoprecipitated with polyclonal antibodies to either α -catenin or β -catenin (both kindly donated by David Rimm, Yale University). Western blots were performed first with the cadherin-11 monoclonal antibody (Fig. 5a), then with a pancadherin polyclonal antibody (Fig. 5b), then with a monoclonal antibody to E-cadherin (Fig. 5c), and finally with antibodies to α -catenin (polyclonal) or β -catenin (monoclonal) (Fig. 5, d and e). Immunoprecipitation with nonimmune rabbit IgG revealed no immunoreactive bands in any of the Western blots (data not shown). The Western blots confirmed that the same five cell lines express cadherin-11, and that in all five cases cadherin-11 is associated with both α -catenin and β -catenin (except in MDA-MB-157 cells, which lack α -catenin). Pancadherin blotting and E-cadherin blotting revealed that several cell lines express other cadherins that are α -catenin and/or β -catenin associated. Several invasive cells express N-cadherin, whereas E-cadherin expression is restricted to the most differentiated, noninvasive cells (12, 20). A few of these cell lines lack α -catenin or β -catenin (See Table 1). One particularly striking example cell line is T47D. In these cells, both α -catenin and β -catenin are

associated with E-cadherin but not with each other. Nevertheless, these cells are well differentiated and exhibit strong cell-cell adhesion, suggesting that compensatory mechanisms exist in the function of the adherens junction (12). In addition to confirming the results of the Western blots, the α -catenin and β -catenin immunoprecipitations identify other defects in the cadherin/catenin system in various cell lines, which are summarized in Table 1. Finally, some proteins that were undetectable by Western blot of cell lysates were present and detected by immunoprecipitation, presumably as a result of the selective concentration afforded by the immunoprecipitation.

Previously, we published a table that listed the molecular characteristics of several breast cancer cell lines (12). We have now updated that table to include the information on cadherin-11 as well as published results on N-cadherin protein and our preliminary results on N-cadherin mRNA. Note in particular that cadherin-11 is expressed only in the cell lines with a stellate morphology. These are the most invasive cell lines, and they all lack E-cadherin.

DISCUSSION

Cadherin-11 Is Expressed in Invasive, Fibroblastoid Breast Cell Lines. Loss of E-cadherin expression or function is associated with a more invasive, less differentiated phenotype in cancer cell lines

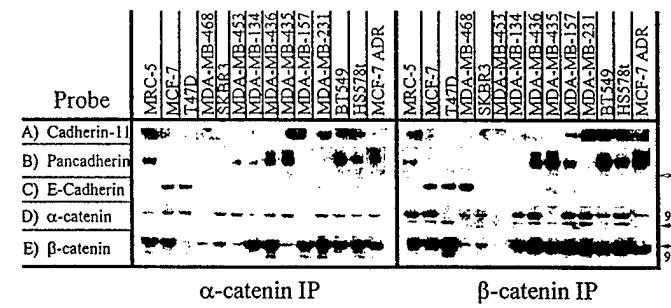


Fig. 5. Immunoprecipitations. Confluent cells were lysed in an NP40 buffer, and the lysate was clarified by microcentrifugation. The lysate was precleared with normal rabbit serum and protein A-Sepharose. Next, 3 μ l of a polyclonal rabbit serum raised against either α -catenin (left) or β -catenin (right) were used for the immunoprecipitations. Immunoprecipitations with 10 μ g of normal rabbit IgG precipitated no immunoreactive bands (data not shown). The immunoprecipitation was run on SDS-PAGE, and the subsequent blot was probed with the following antibodies: A, cadherin-11 monoclonal antibody; B, pancadherin polyclonal antibody; C, E-cadherin monoclonal antibody; D, α -catenin polyclonal; and E, β -catenin monoclonal antibody. The open arrow marks the location of the cadherin-11 band on the pancadherin blot.

and primary tumor samples (11, 12). In earlier studies, we established three subsets from a panel of breast cancer cell lines (11, 12). E-cadherin-expressing cell lines (which all lack vimentin) were poorly invasive in Boyden Chamber assays and well-differentiated, forming tight cell clusters, in Matrigel. Cell lines lacking E-cadherin and vimentin were also poorly invasive but only moderately differentiated, forming loose cell aggregates in Matrigel. Finally, cell lines lacking E-cadherin but expressing vimentin were highly invasive and poorly differentiated and had a stellate, fibroblastoid morphology. However, transfection of E-cadherin into these cells did not reverse the invasive phenotype (12). These results indicated that loss of E-cadherin expression or function may be necessary but is not sufficient for the establishment of invasive, highly malignant tumors.

Despite the lack of E-cadherin expression, most of the invasive cells exhibited calcium-dependent adhesion, indicating the presence of another member of the cadherin family (11). Recent work has demonstrated the expression of cadherins in fibroblastic and mesenchymal cells. N-cadherin is expressed in several types of mesenchymal tissue as well as in cultured fibroblasts (14). In addition, cadherin-11 expression is restricted to mesenchymal cells in the developing mouse (13, 15, 22) and is expressed in stromal cells in the adult (19). Hazan *et al.* (20) have shown that N-cadherin expression in breast cancer cell lines is limited to most of the invasive, fibroblastoid cells. They proposed that N-cadherin expression is restricted to less differentiated cells, and that the expression of N-cadherin facilitates the interaction of tumor cells with the underlying stroma. Our preliminary results indicate that N-cadherin mRNA (but not a pan-cadherin immunoreactive protein) is also present in several noninvasive cell lines (see Table 1; data not shown). Consequently, it may be interesting to explore the posttranscriptional regulation of N-cadherin and its possible relevance in breast cancer.

We now present evidence that cadherin-11 is expressed in breast cancer cell lines that lack E-cadherin but express vimentin and are highly invasive and poorly differentiated. RT-PCR, Northern blot, and Western blot analyses confirm that five of seven invasive breast cancer cell lines express cadherin-11. Cadherin-11 was found previously to be expressed only in embryonic mesenchymal tissues, osteoblasts, and invasive tumors of the stomach and the kidney (17, 19, 22, 23). Furthermore, cadherin-11 has never been shown to be expressed in E-cadherin-expressing cell lines or tissues (22, 23). This indicates that cadherin-11 is a very specific marker for only the most invasive subset of cancer cell lines. Although we have been unable to determine the level of expression of cadherin-11 in human tumor tissue, the results obtained using cancer cell lines suggest that cadherin-11 is a potential molecular marker and could be used to identify highly malignant tumors that would require more aggressive therapy. Moreover, our results, together with those of Hazan *et al.* (20), show that invasive cells express cadherin-11, N-cadherin, or both and indicate that detection of these molecules could identify with confidence highly malignant tumors.

The five cell lines that express cadherin-11 also express a variant of cadherin-11. This variant arises from a 179-bp insertion that results in a protein that lacks the majority of the wild-type cytoplasmic domain, including the catenin-binding regions (17). If the cadherin-11 variant is expressed as a functional protein, it could act as a dominant negative and reduce cadherin-mediated cell adhesion. In fact, expression of this variant mRNA has been associated with invasive tumors (17, 24). However, by using cell extracts, we were unable to definitively identify a protein product of a size anticipated from the variant mRNA sequence.

Cadherin-11 Associates with α - and β -Catenin. Cadherin-11 is a typical type II cadherin and can mediate calcium-dependent cell-cell adhesion. Previously, Shibata *et al.* (19) have shown that cadherin-11

interacts with β -catenin *in vitro*. We have shown here that cadherin-11 forms complexes containing both α - and β -catenin *in vivo*. However, in the invasive breast cancer cell, BT549 cadherin-11 is found predominantly in a detergent-soluble pool, indicating that it is not associated with the cytoskeleton. In the same cells, a pan-cadherin reactive protein (probably N-cadherin) is found in the detergent insoluble pool. Thus, cadherin-11 is not found in a typical adherens junction in which a cadherin is linked, through the catenins, to the actin cytoskeleton. Adherens junctional cadherins are also localized to sites of cell-cell contact. However, our immunocytochemistry shows that cadherin-11 is also found at lamellipodia-like extensions and possibly to regions of cell-substrate contact. These results suggest that cadherin-11 may have several functions. It may function in a transient form of cell-cell adhesion that involves the cytoplasmic catenins but is not associated with the cytoskeleton. The cadherin-11 complex may also have a role in contacting the cell-matrix, particularly in leading extensions of the cell. Such a complex would be much more transient and could facilitate the ability of a motile cell to interact with its surroundings, which could include both matrix proteins, as well as other mesenchymal cells. These potential functions of cadherin-11 have implications with respect to the invasive and metastatic ability of tumor cells.

Functional Significance of Cadherin-11. Cadherin-11 could be involved in cell and matrix recognition that may facilitate cell motility and may also be essential for the loose aggregation of cell types that is necessary in tissue morphogenesis. These hypotheses are supported by several observations. First, cadherin-11 expression is associated with invasive cells, both during normal stages of embryogenesis and in invasive tumor cells (17, 19, 22, 25). This invasion may be facilitated by the association of the invasive cadherin-11 cells with the surrounding mesenchymal cells, which also express cadherin-11, as well as with the surrounding matrix. For example, during embryogenesis mesenchymal cells express cadherin-11 (15). In addition, cells that undergo an epithelial to mesenchymal transition also begin to express cadherin-11 as they invade the surrounding tissue to form new structures (22). This can be seen during branching morphogenesis of lungs and kidneys and also in the formation of the nasal septum, skin, vibrissae, teeth, and various glands (22). Shibata *et al.* (19) show that in a panel of gastric tumors (both primary tumors and tumor cell lines), cadherin-11 is only expressed in signet ring cell carcinomas, which are typically infiltrative. They suggest that the expression of cadherin-11 in the tumor cells may allow for interactions with the underlying stroma that would facilitate invasion. Thus, unlike other cadherins, such as E-cadherin and cadherin-6, which have invasion suppressor function, cadherin-11 may actually enhance tumor cell invasiveness and may be a new target for treatment (11).

Next, cadherin-11 may be essential for the loose aggregation of cell types. For example, during embryogenesis, the expression of cadherin-11 is increased dramatically in cells undergoing the epithelial-mesenchymal transition that precedes the formation of various structures and also in regions of mesenchymal condensation, such as occurs prior to chondrogenesis (13). The association and loose aggregation of cells may depend upon cadherin-11 and could be extremely significant in cancer. For example, it is possible that cadherin-11 may act to specifically target metastatic tumor cells to sites that express cadherin-11. Consequently, expression of cadherin-11 may facilitate association of metastatic cells with cadherin-11-expressing osteoblasts in the bone, thus establishing a bony metastasis.

These studies indicate that cadherin-11 expression is associated with invasive breast cancer and may play a significant role in facilitating tumor cell invasion and the formation of metastatic tumors. Elucidation of the functions and regulation of cadherin-11 may enhance our understanding of the roles of cadherins in invasive cancer and mesenchymal cells and may provide future targets for therapy.

ACKNOWLEDGMENTS

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